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(54) Title: METHOD FOR THE PRODUCTION OF D-GLUCONIC ACID

(57) Abstract

A process for producing D-gluconic acid, which comprises cultivating a microorganism belonging to the genus Bacillus, which is capable of producing D-gluconic acid from D-glucose, which lacks gluconokinase activity, and which has high glucose dehydrogenase activity, in the presence of D-glucose, in a culture medium and recovering the resulting D-gluconic acid from the culture broth.

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Method for the production of D-gluconic acid

This invention relates to a method for producing D-gluconic acid from D-glucose by fermentation.

D-gluconic acid and its derivatives have many commercial uses, such as being agents for the regulation of solidifying concrete, agents in textile printing and textile bleaching, as agents for preventing milkstone and beerstone in the dairy industry and breweries, respectively, and as sequestrant. D-gluconic acid and its derivatives have also a wide use in food and pharmaceutical industry and in detergents.

Many fermentation processes for the production of D-gluconic acid are known. Many microorganisms, such as Acetobacter, Pseudomonas, Gluconobacter, Aspergillus and Penicillium are known to be able to accumulate D-gluconic acid. However, the known fermentation processes are not fully satisfactory as commercial processes for the production of D-gluconic acid in terms of yields.

According to the present invention, it is possible to produce D-gluconic acid at a satisfactorily high yield, i.e. as a commercial process by using the mutants of bacteria of the genus Bacillus. It has been found that mutants of bacteria of the genus Bacillus which lack gluconokinase activity and have high glucose dehydrogenase activity have an unusually high ability to accumulate D-gluconic acid. The present invention has been accomplished based on this finding.

The microorganisms belonging to the genus Bacillus exhibit several advantages. The fermentation process by the genus Bacillus is simple because

such microorganism can grow fast even in a simple and cheap medium, and because it is easy to handle, for instance, in the preparation of seed culture, and in the removal of the bacterial cells in the fermentated broth. In addition, the isolation procedure for gluconic acid from the broth is easy, due to little accumulation of other organic acids, which may cause difficulty in separation, unlike gluconic acid. Moreover, the genus Bacillus has high stability in genetic characteristics, thus it can be stocked easily without decrease of its activities for the production.

The present invention is thus concerned with a process for producing D-gluconic acid which comprises cultivating a microorganism belonging to the genus Bacillus, which is capable of producing D-gluconic acid from D-glucose, which lacks gluconokinase, and which has high glucose dehydrogenase activity, in the presence of D-glucose in a culture medium, and recovering the resulting D-gluconic acid from the culture broth.

In this context, the lack of gluconokinase activity means, for example, that when, by the following method, the discipline of which is described in Biochim. Biophys. Acta. 798, 88-95 (1984), the amount of reduction of oxidation form of nicotinamide adenine dinucleotide phosphate (hereinafter referred to as NADP) is measured and the particular enzymatic activity is calculated for said cell free extract prepared by the procedure as described below (See Example 3),the value is not more than 0.001 unit/mg-protein.

Procedure for Assay of Gluconokinase

The reaction mixture (0.5 ml) contained 100 µmole of Tris-HCl buffer (pH 8.0), 6.6 µmole of $MgCl_2$, 3.2 µmole of adenosine triphosphate, 0.4 µmole of NADP, 1.0 µmole of sodium gluconate, 20 µl of the cell free extract and 0.005 unit of authentic 6-phosphogluconate dehydrogenase (Enzyme code 1.1.1.44, Sigma Chemical Co.,Ltd.). The reaction was initiated by the addition of the substrate. The change of absorbance at 340 nm was measured with a spectrophotometer Model UVIKON 810 (Kontron K.K.) at room temperature. One unit of the enzyme activity was defined as the amount of the enzyme catalyzing the reduction of 1 µmole of NADP per minute.

In this context, high glucose dehydrogenase activity means, for example, that when, by the following method, the discipline of which is described in Agric. Biol. Chem. 43, 271-278 (1979), the amount of reduction of NADP is measured at 340 nm with a spectrophotometer Model UVIKON 810, and the

particular enzymatic activity is calculated for said cell free extract as described above, the value is not less than 0.1 unit/mg-protein.

Procedure for Assay of Glucose Dehydrogenase

The assay mixture (0.5 ml) contained 50 µmole of D-glucose, 2 µmole of NADP, 0.3 mmole of Tris-HCl buffer(pH 8.0), 5 nmole of $MnSO_4$ and 20 µl of the cell free extract. The reaction was initiated by the addition of the substrate. One unit of enzyme activity was defined as the amount of enzyme catalyzing the reduction of 1 µmole of NADP per minute.

The microorganisms used in the present invention embrace all the strains belonging to the genus Bacillus which lack gluconokinase and have high glucose dehydrogenase activity. Such strains can be easily derived from microorganisms belonging to the genus Bacillus such as , Bacillus brevis, Bacillus cereus, Bacillus circulans, Bacillus coagulans, Bacillus licheniformis, Bacillus megaterium, Bacillus mesentricus, Bacillus pumilus, Bacillus subtilis, etc., by such means as irradiating the parent strains with radiation such as ultraviolet light, X-rays, gamma rays or the like, or exposing the parent strains to the action of chemical mutagens, such as N-methyl-N'-nitro-N-nitrosoguanidine (hereinafter referred to as MNNG), nitrogen mustard, ethylmethanesulfonate, etc.

Examples of the strains most preferably used in the present invention are Bacillus pumilus RMXI and the like. This microorganism has been deposited in Agency of Industrial Science and Technology, Fermentation Research Institute, Japan under the following Number:

Bacillus pumilus RMXI FERM-BP No. 3335
(date of deposit: March 29, 1991)

In the preferable embodiment of the present invention, the production of D-gluconic acid is effected by cultivating the above microorganism in an aqueous medium containing D-glucose and supplemented with appropriate nutrients under aerobic conditions. Said medium can contain D-glucose in a concentration of about 50 g/l to about 300 g/l, preferably from about 100 g/l to about 250 g/l.

It is usually required that the culture medium contains nutrients as assimilable carbon sources, e.g. D-glucose, D-fructose, D-mannose, D-sorbitol, D-mannitol, sucrose, molasses, starch hydrolyzates, starch, acetic acid and

ethanol; digestible nitrogen sources such as organic substances, for example, peptone, yeast extract, soybean meal, corn steep liquor, cottonseed refuse, dried yeast and meat extract, and inorganic substances, for example, ammonium sulfate, ammonium chloride, ammonium phosphate, potassium nitrate and potassium phosphate; vitamins, metals, amino acids and trace elements, etc.

The cultivation may be conducted at pH values of about 4.0 to about 9.0, preferably from about 4.5 to about 8.0. A cultivation period varies depending upon the microorganisms and nutrient medium to be used, preferably about 10 to about 150 hours. A preferred temperature range for carrying out for the cultivation is from about 20 to about 45°C, preferably from about 25 to about 40°C.

It is also possible to use immobilized microorganism on appropriate supports, such as K-carrageenan, calcium alginate and other polymers, for the production of D-gluconic acid, and this enables the microorganism to be used repeatedly.

The D-gluconic acid thus accumulated can be easily recovered, for example, by the following procedure:

The culture broth is first adequately diluted with water to dissolve D-gluconic acid, which has precipitated in the culture broth, then filtered or centrifuged, whereby the cells can be removed with great ease. Then, the filtrate might be decolorized, e.g. by treatment with activated carbon, and then, concentrated. To the concentrate is added an appropriate organic solvent, such as ethanol, whereupon D-gluconic acid crystals separate in the salt form, such as the sodium salt and the calcium salt, for example. Whether the above or any other appropriate and known method, e.g. for the separation of D-gluconic acid, e.g. out of a culture broth is employed, D-gluconic acid can always easily be recovered.

EXAMPLES

The following examples are further illustrative of this invention, it being understood, however, that the invention is by no means limited thereto.

Preparation of the Mutant Strain RMX1

One loopful of Bacillus pumilus RE5 (FERM-BP NO. 2833) grown on an agar medium was inoculated into a 100 ml of seed culture medium whose composition is shown below.

D-Sorbitol	2 %
Corn steep liquor	2 %
KH_2P0_4	0.1 %
K_2HP0_4	0.3 %
L-Phenylalanine	0.0025 %
L-Tryptophan	0.0025 %
(pH 6.7 before sterilization)	

The flask was incubated at 30°C for 18 hours. The cells were collected by centrifugation and suspended into 20 ml of 50 mM phosphate buffer (pH 8.0). A portion of the cell suspension (0.75ml) was added by 100 µg/ml (final concentration) of MNNG and treated for 30 minutes at 30°C. The treated cells were collected by centrifugation, washed once by sterile water, resuspended into 5 ml of seed culture medium and incubated for 2 hours at 30°C. The culture thus prepared was appropriately diluted by sterile water and spread on the agar culture medium as shown below.

D-Glucose	18.0 %
Molatein (dried yeast, Kanegafuchi Kagaku)	0.07 %
D-Sorbitol	0.2 %
Corn steep liquor	0.5 %
$(NH_4)_2S0_4$	0.55 %
$(NH_4)_2HP0_4$	0.2 %
KH_2P0_4	0.01 %
K_2HP0_4	0. 03 %
L-Phenylalanine	0.00025 %
L-Tryptophan	0.00025 %
$FeS0_47H_20$	0.00032 %
$MnS0_46H_20$	0.00019 %
$CaC0_3$	4 %
Agar	1.5 %

The plates were incubated at 36.5°C for 2 days. Colonies well grown on the plates were streaked on a fresh agar medium as shown below.

D-Sorbitol	0.5 %
Bacto peptone (Difco)	1 %
Yeast extract	

(Oriental Yeast)	0.2%
NaCl	0.2%
Agar	1.5 %

(pH 7.0)

The plates were incubated at 36.5°C for 24 hours to obtain enough amount of cell mass for tube culture as described below.

Each of the agar cultures thus prepared was used to inoculate 5 ml of production medium as shown below.

D-Glucose	18.0 %
Molatein (dried yeast, Kanegafuchi Kagaku)	0.07 %
D-Sorbitol	0.2 %
Corn steep liquor	0.5 %
$(NH_4)_2S0_4$	0.55 %
$(NH_4)_2HP0_4$	0.2 %
KH_2P0_4	0.01 %
K_2HP0_4	0. 03 %
L-Phenylalanine	0.00025 %
L-Tryptophan	0.00025 %
$FeS0_47H_20$	0.00032 %
$MnS0_46H_20$	0.00019 %
$CaC0_3$	4 %

The tubes were incubated for 5 days at 36.5°C. Then the supernatant of the fermented broth was obtained by centrifugation and analyzed for D-gluconic acid production level by thin layer chromatography. One µl of the supernatant was spotted on a silica gel plate (Kieselgel $60F_{254}$, Merck) and developed by a solvent system consisting of n-propanol, 95% ethanol, and 0.05M potassium phosphate buffer (55:25:20) . Then silica gel plates were sprayed with $KI0_4$-Tetrabase reagent to visualize the spot of D-gluconic acid. Out of the mutants cultivated, the strain RMXI was selected as a D-gluconic acid high producer which was superior to its parent strain.

EXAMPLE 1

One loopful of agar culture of Bacillus pumilus ATCC 31093 (the parent strain of RE5), the parent strain RE5 and the mutant RMX1 were inoculated into 5 ml of production medium in test tubes, respectively. The composition of the medium is shown below.

D-Glucose	18.0 %
Molatein (dried yeast, Kanegafuchi Kagaku)	0.07 %
D-Sorbitol	0.2 %
Corn steep liquor	0.5 %
$(NH_4)_2SO_4$	0.55 %
$(NH_4)_2HPO_4$	0.2 %
KH_2PO_4	0.01 %
K_2HPO_4	0.03 %
L-Phenylalanine	0.00025 %
L-Tryptophan	0.00025 %
$FeSO_47H_20$	0.00032 %
$MnSO_46H_20$	0.00019 %
$CaCO_3$	4 %

The tubes were incubated at 36.5°C for 5 days. The D-gluconic acid productivities of Bacillus pumilus ATCC 31093, the parent strain RE5 and the mutant RMX1 are shown in Table 1. The mutant RMX1 showed about 16 and 37 times higher productivity of D-gluconic acid than Bacillus pumilus ATCC 31093 and the parent strain RE5, respectively.

Table 1

D-gluconic acid Productivity of Bacillus pumilus ATCC 31093, RE5 and RMX1

Strain	D-gluconic acid produced (g/l)* in 5 days
Bacillus pumilus ATCC 31093	9.7
Bacillus pumilus RE5	4.1
Bacillus pumilus RMX1	153.2

* Values are expressed in terms of the free form. (D-gluconic acid was actually accumulated as calcium salt in the culture broth.)

EXAMPLE 2

One loopful of agar culture of Bacillus pumilus ATCC 31093, the parent strain RE5 and the mutant RMX1 were inoculated into 7 ml of seed culture medium in test tubes, respectively. The composition of the medium is shown below.

D-Sorbitol	2.0 %
Corn steep liquor	2.0 %
KH_2PO_4	0.1 %
K_2HPO_4	0.3 %
L-Phenylalanine	0.0025 %
L-Tryptophan	0.0025 %

(pH 6.7)

The inoculated test tubes were incubated at 36.5°C for 6 hours on a tube shaker. The seed cultures thus prepared (4 ml) were inoculated into production media made up to 40 ml after inoculation in 500 ml Erlenmeyer flasks. The composition of the production medium was as follows.

Molatein	0.07 %
$(NH_4)_2SO_4$	0.55 %
$(NH_4)_2HPO_4$	0.2 %
$FeSO_47H_20$	0.00032 %
$MnSO_46H_20$	0.00019 %
$CaCO_3$	5.0 - 7.0 %
D-Glucose	(See: Table 2)
Corn steep liquor	0.45 %

(pH ca.7.0)

The flasks were incubated at 36.5°C and 220 rpm for 6 days. The D-gluconic acid productivities of Bacillus pumilus ATCC 31093 of the strain RE5 and the mutant RMX1 are shown in Table 2. The mutant RMX1 showed much higher productivity of D-gluconic acid than did Bacillus pumilus ATCC 31093 or the strain RE5. The mutant RMX1 produced 217.6 g/l of D-gluconic acid

from 250 g/l of D-glucose (molar conversion, yield = 80 %) in 6 days fermentation.

From this fermentation broth, the cells were removed by filtration after the broth was diluted with water to dissolve D-gluconic acid which was precipitated in the culture broth, and the filtrate was concentrated to half the original volume. Then, about one-quarter of its volume of ethanol was added and the precipitate was discarded. The supernatant was decolorized on a column of activated carbon. The decolorized solution was concentrated, and about 4 times its volume of ethanol was added, whereby 7.3 g of crystalline D-gluconic acid (calcium salt) was obtained (96 % purity).

Table 2

D-gluconic acid productivity of Bacillus pumilus ATCC 31093, RE5 and RMX1

Strain	D-glucose (g/l)	D-gluconic acid producted in 4 days	6 days
Bacillus pumilus ATCC 31093	180	23.3 g/l * (11.9 %) **	
Bacillus pumilus RE5	180	3.4 (1.7)	8.6 (4.4)
	200	13.1 (6.0)	11.0 (5.1)
	220	24.6 (10.3)	28.6 (11.9)
	250	1.7 (0.6)	3.0 (1.1)
Bacillus pumilus RMX1	180	168.9 (86.2)	165.5 (84.4)
	200	175.4 (80.5)	173.6 (79.7)
	220	201.8 (84.2)	203.8 (85.1)
	250	167.3 (61.5)	217.6 (80.0)

* Values are expressed in terms of the free form. (D-gluconic acid was accumulated as calcium salt in the culture broth.)

** Molar conversion yield (%)

Example 3

In the same manner as described in Example 2, Bacillus pumilus ATCC 31093, the parent strain RE5 and the mutant RMXI were cultivated in 500 ml Erlenmeyer flasks . When the D-glucose in medium had completely disappeared - as confirmed by a test-paper normally used for urine sugar analysis, 50 ml of cultured broth was withdrawn from each flask . The broth was centrifuged at 6,000 x g for 10 minutes, and the precipitated cells were suspended in 10 ml of 50 mM Tris-HCl buffer(pH 7.5). The cell suspension was centrifuged at 6,000 x g for 10 minutes, and the precipitated cells were washed again by the same procedure as described above. The obtained cells were frozen at -20°C until use.

The frozen cells were thawed, suspended in 10 ml of 50 mM Tris-HCl buffer (pH 7.5) and centrifuged at 6,000 x g for 10 minutes. The precipitated cells were re-suspended in 10 ml of the same buffer and added by lysozyme (Sigma Chemical Co.) to the final concentration of 500 μg/ml. The mixture was then incubated at 37°C for 1 hour with agitation (240 rpm) to lyse the cells. The lysate thus obtained was centrifuged at 6,000 x g for 10 minutes. The resulting supernatant was used as cell free extract.

The enzyme activities of glucose dehydrogenase (GDH) and gluconokinase (GAK) in the cell free extracts of Bacillus pumilus ATCC 31093 and the parent RE5 and the mutant RMXI were measured. The results are shown in Table 3. It was found that the mutant RMXI possessed about 4.5 times higher specific activity of glucose dehydrogenase than the parent.

Table 3

Enzyme Activities of Cell Free Extracts of Bacillus pumilus ATCC31093, RE5 and RMX1

	Enzyme Activity of		
Enzymes	B.pumilus ATCC 31903	B.pumilus RE5	B.pumilus RMX1
GDH	0.065 * (0.026) **	0.098 (0.051)	0.143 (0.229)
GAK	0.009 (0.004)	0.073 (0.029)	0 (0)

* unit / ml-broth
** unit / mg-protein

Example 4

In the same manner as described in Example 2, the seed culture of the mutant RMX1 was prepared and inoculated into the production medium whose composition was as follows.

Molatein	0.5%
Corn steep liquor	0.45%
$(NH_4)_2SO_4$	0.55%
$(NH_4)_2HPO_4$	0.2%
$FeSO_47H_2O$	0.00032%
$MnSO_4 \cdot 6H_2O$	0.00019%
D-Glucose	25%

The flask was incubated at 36.5°C and 240 rpm for 4 days. 5% of D-glucose (4 ml of 50% solution) was added after 3 days of cultivation. As a result, the mutant RMX1 produced 303.4 g/l of D-gluconic acid from a total 30% of D-glucose (molar conversion, yield = 93%) after 4 days fermentation.

Example 5

One loopful of agar culture of Bacillus pumilus RMX1 was inoculated into 100 ml of seed culture medium in 500 ml Erlenmeyer flasks. The composition of the medium is shown below.

D-Sorbitol	6.0%
Corn steep liquir	6.0%
KH_2PO_4	0.3%
K_2HPO_4	0.9%
L-Phenylalanine	0.0075%
L-Tryptophan	0.0075%

(pH 6.7)

The inoculated flasks were incubated at 36.5°C for 7.5 hours. The seed culture thus prepared (300 ml) was inoculated into the production medium made up to 3 L after inoculation in a 5 L jar fermentor (B.E. Marubishi Co., Ltd.). The composition of the production medium was as follows.

Molatein	0.5%
Corn steep liquor	0.9%
$(NH_4)_2SO_4$	0.55%
$(NH_4)_2HPO_4$	0.2%
$FeSO_47H_2O$	0.00032%
$MnSO_4 \cdot 6H_2O$	0.00019%
D-Glucose	25%

The fermentation was carried out at 36.5°C, with agitation at 500 rpm and aeration at 0.5 vvm. The pH value was controlled with 6N NaOH (not to be lowered below 5.3).

As a result, 229.3 g/l of D-gluconic acid (255.0 g/l of Na-D-gluconic acid) was produced from 246.6 g/l of D-glucose (molar conversion, yield = 85.4%).

Claims

1. A process for producing D-gluconic acid, which comprises cultivating a microorganism belonging to the genus Bacillus, which is capable of producing D-gluconic acid from D-glucose, which lacks gluconokinase activity, and which has high glucose dehydrogenase activity, in the presence of D-glucose, in a culture medium, and recovering the resulting D-gluconic acid from the culture broth.

2. A process according to claim 1, wherein the microorganism belonging to the genus Bacillus has at least 0.1 units of glucose dehydrogenase activity/mg protein.

3. A process according to claim 1, wherein the corresponding micro-organism has less than 0.001 units of gluconokinase activity/mg protein.

4. A process according to claim 1, wherein the microorganism is of the species Bacillus brevis, Bacillus cereus, Bacillus circulans, Bacillus coagulans, Bacillus licheniformis, Bacillus megaterium, Bacillus mesentricus, Bacillus pumilus and Bacillus subtilis.

5. A process according to claim 1, wherein the microorganism is Bacillus pumilus RMX1 (FERM BP-3335).

6. A process according to any one of claims 1 - 5, wherein the initial concentration of D-glucose in the culture medium is about 50 g/l to about 300 g/l, preferably from about 100 g/l to about 250 g/l.

7. A process according to any one of claims 1 - 6, wherein the cultivation is carried out at a pH between about 4.0 and 9.0, preferably between about 5.0 and 8.0.

8. A process according to any one of claims 1 - 7, wherein the cultivation is carried out at a temperature between about 20 and 45°C, preferably between about 25 and 40°C.

9. Bacillus pumilus RMX1 (FERM-BP No. 3335) which is capable of producing D-gluconic acid from D-glucose, which Bacillus lacks gluconokinase activity and has high glucose dehydrogenase activity.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 92/00775

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12P7/18; C12N1/20; //(C12N1/20;C12R1:07)
(C12P7/18;C12R1:07)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12P ; C12R

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	CHEMICAL ABSTRACTS, vol. 105, no. 7, 18 August 1986, Columbus, Ohio, US; abstract no. 57740G, OTANI,MIEKO ET AL: 'Predominance of gluconate formation from glucose during germination of Bacillus megaterium' page 353 ;column R ; see abstract & JOURNAL OF BACTERIOLOGY vol. 167, no. 1, 1986, pages 148 - 152; --- -/--	1-4,6-8

[10] Special categories of cited documents:

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
05 JUNE 1992	[illegible]
International Searching Authority	**Signature of Authorized Officer**
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	CHEMICAL ABSTRACTS, vol. 92, no. 23, 9 June 1980, Columbus, Ohio, US; abstract no. 194200H, MARUYAMA,TOMOKI ET AL: 'Glucose catabolism during germination of Bacillus megaterium spores' page 325 ;column R ; see abstract & JOURNAL OF BACTERIOLOGY vol. 141, no. 3, 1980, pages 1443 - 1446; ---	1-4,6-8
A	CHEMICAL ABSTRACTS, vol. 107, no. 7, 17 August 1987, Columbus, Ohio, US; abstract no. 55522E, OTANI,MIEKO ET AL: 'Gluconate metabolism in germinated spores of Bacillus megaterium QM B1551:primary roles of gluconokinase and the pentose cycle' page 417 ;column R ; see abstract & BIOCHIMICA ET BIOPHYSICA ACTA vol. 924, no. 3, 1987, pages 467 - 472; ---	1-4,6-8
A	CHEMICAL ABSTRACTS, vol. 104, no. 13, 31 March 1986, Columbus, Ohio, US; abstract no. 103309C, FUJITA,YASUTARO ET AL: 'The characterization and cloning of a gluconate -gnt- operon of Bacillus subtilis' page 158 ;column L ; see abstract & J.GEN.MICROBIOL. vol. 132, no. 1, 1986, pages 161 - 169; ---	1-4,6-8
A	BIOCHIMICA ET BIOPHYSICA ACTA vol. 798, 1984, ELSEVIER ;AMSTERDAM,NL. pages 88 - 95; Y. FUJITA ET AL: 'Catabolite repression of inositol dehydrogenase and gluconate kinase synthese in Bacillus subtilis' cited in the application see the whole document ---	1-4,6-8

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	CHEMICAL ABSTRACTS, vol. 92, no. 23, 9 June 1980, Columbus, Ohio, US; abstract no. 194200H, MARUYAMA,TOMOKI ET AL: 'Glucose catabolism during germination of Bacillus megaterium spores' page 325 ;column R ; see abstract & JOURNAL OF BACTERIOLOGY vol. 141, no. 3, 1980, pages 1443 - 1446; ---	1-4,6-8
A	CHEMICAL ABSTRACTS, vol. 107, no. 7, 17 August 1987, Columbus, Ohio, US; abstract no. 55522E, OTANI,MIEKO ET AL: 'Gluconate metabolism in germinated spores of Bacillus megaterium QM B1551:primary roles of gluconokinase and the pentose cycle' page 417 ;column R ; see abstract & BIOCHIMICA ET BIOPHYSICA ACTA vol. 924, no. 3, 1987, pages 467 - 472; ---	1-4,6-8
A	CHEMICAL ABSTRACTS, vol. 104, no. 13, 31 March 1986, Columbus, Ohio, US; abstract no. 103309C, FUJITA,YASUTARO ET AL: 'The characterization and cloning of a gluconate -gnt- operon of Bacillus subtilis' page 158 ;column L ; see abstract & J.GEN.MICROBIOL. vol. 132, no. 1, 1986, pages 161 - 169; ---	1-4,6-8
A	BIOCHIMICA ET BIOPHYSICA ACTA vol. 798, 1984, ELSEVIER ;AMSTERDAM,NL. pages 88 - 95; Y. FUJITA ET AL: 'Catabolite repression of inositol dehydrogenase and gluconate kinase synthese in Bacillus subtilis' cited in the application see the whole document ---	1-4,6-8